(12) **UK Patent Application** (19) **GB** (11) **2 246 569** (13)**A**

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(54) Tumour necrosis factor - alpha binding protein

(57) A protein or polypeptide which has the amino acid sequence of the extracellular domain of human TNF-α receptor, and in particular polypeptide having the amino acid sequence (I):

LVPHLGDREKRDSVCPQGKYIHPQNNSICCTKCHKGTYLYNDCPGQDTDCRECESGS
FTASENHLRHCLSCSKCRKEMGQVEISSCTVDRDTVCGCRKNQYRHYWSENLFQCFN
CSLCLNGTVHLSCQEKQNTVCTCHAGFFLRENECVSCSNCKKSLECTKLCLPQI, or a
derivative thereof to which human TNFα is capable of binding and whose amino acid sequence has a degree of homology of 90% or more with the sequence (I). The above amino acid sequence may be modified by removal of the first 11 aminoterminal residues and by extension at the carboxyl end with E N V K G T E D S G T T. The DNA sequence for polypeptide (I) is also given.

The protein is useful in the treatment of rheumatoid arthritis.

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Fig.1.

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1   ACCA GTGATCTCTA TGCCCGAGTC TCAACCCTCA ACTGTCACCC CAAGGCACTT GGACGTCCT GGACAGACCG
75  AGTCCCGGGA AGCCCCAGCA CTGCCGCTGC CACACTGCCC TGAGCCCAAA TGGGGGAGTG AGAGGCCATA GCTGTCTGGC

40    M   G   L   S   T   V   P   D   L   L   L   P   L   L   E   L   L   V   G   I   Y   P
156   ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTC CTG GAG CTG CTG GTG GGA ATA TAC CCC

16    S   G   V   I   L   V   P   H   L   G   D   R   E   K   D   S   V   C   P   Q   G   K
228   TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG GAT AGT GTG TGT CCC CAA GGA AAA

9     Y   I   H   P   Q   N   N   S   I   C   T   K   C   H   K   G   T   Y   L   Y   N   D   C
300   TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC ACC AAG TGC CAC AAG GGA ACC TAC TTG TAC AAT GAC TGT

33    P   G   P   G   Q   D   T   D   C   R   E   C   E   S   G   S   F   T   A   S   E   N   H   L
372   CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC

57    R   H   C   L   S   C   S   K   C   R   K   E   M   G   Q   V   E   I   S   C   T   V   D
444   AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TGT ACA GTG GAC

81    R   D   T   V   C   G   C   R   K   N   Q   Y   H   Y   W   S   E   N   L   F   Q   C   F
516   CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC

105   N   C   S   L   C   L   N   G   T   V   H   L   S   C   Q   E   K   Q   N   T   V   C   T   C
558   AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC

129   H   A   G   F   F   L   R   E   N   E   C   V   C   S   N   C   K   K   S   L   E   C   T
660   CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC AAC TGT AAG AAA AGC CTG GAG TGC ACG

153   K   L   C   L   P   Q   I   E   N   V   K   G   T   E   D   S   G   T   L   L   P   L
732   AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC CTG CTG CCC CTG

177   V   I   F   F   G   L   C   L   L   S   L   L   F   I   G   L   M   Y   R   Y   Q   R   W   K
804   GTC ATT TTC TTT GGT CTT TGC CTC TTA TCC CTC CTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG

201   S   K   L   Y   S   I   V   C   G   K   S   T   P   E   K   E   G   E   L   E   G   T   T   T
876   TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT
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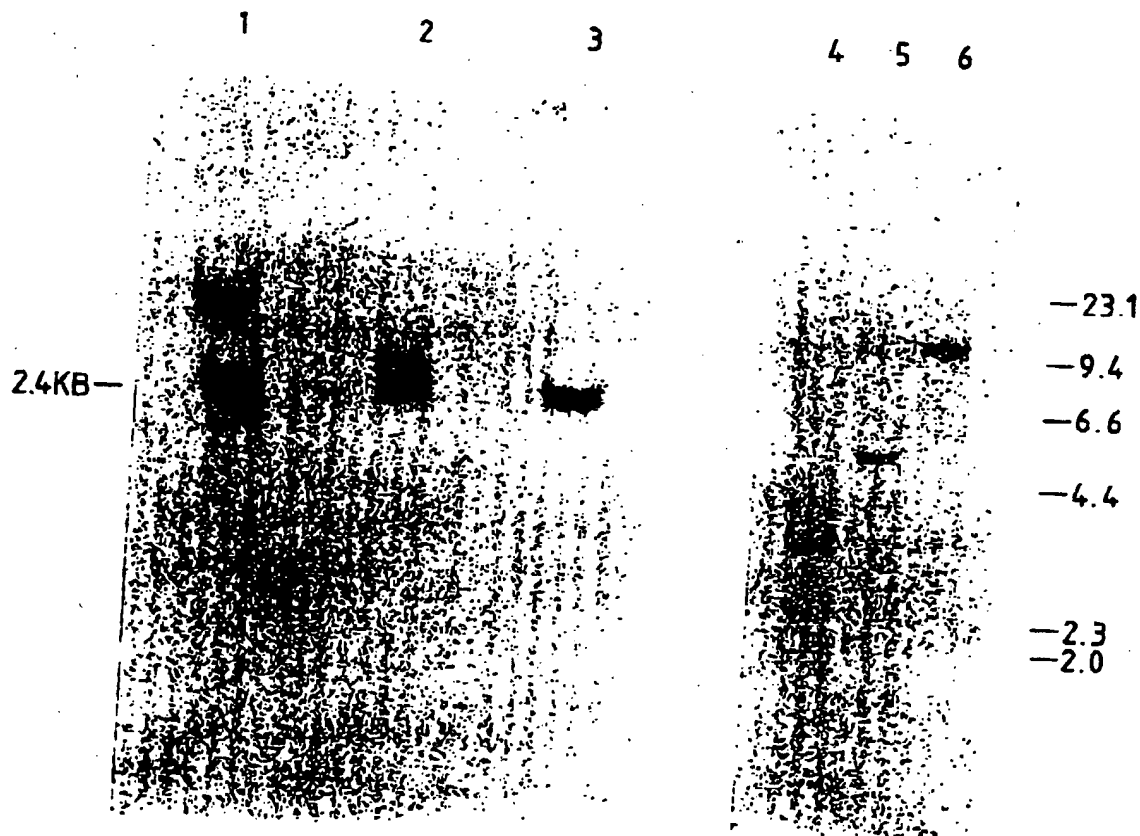
Fig.1(cont.)

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225 K   P   L   A   P   N   P   S   F   S   P   T   P   G   F   T   P   T   L   G   F   T   P   S   P   V
948 AAG CCC CTG GCC CCA AAC CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC CTG TTC AGT CCC GTG

249 P   S   S   T   F   T   S   S   T   Y   T   P   G   D   C   P   N   P   A   A   P   R   R
1020 CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA

273 E   V   A   P   P   Y   Q   G   A   D   P   I   L   A   T   A   L   A   S   D   P   I   P   N
1092 GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GAC TCC GAC CCC ATC CCC AAC

297 P   L   Q   K   W   E   D   S   A   H   K   P   Q   S   L   D   T   D   D   P   A   T   L   Y
1164 CCC CTT CAG AAG TGG GAG GAC AGT GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC

321 A   V   V   E   N   V   P   P   L   R   T   L   E   P   V   R   R   L   G   L   S   D   H   E
1236 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC ACT CTC AAG GAA TTC GTG CGG CGG CTA GGG CTG AGC GAC CAC GAG

345 I   D   R   L   E   L   Q   N   G   R   C   L   R   E   A   Q   Y   S   M   L   A   T   W   R
1308 ATC GAT CGG CTG GAG CTG CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG

369 R   T   P   R   R   E   A   T   L   E   L   L   G   R   V   L   R   N   M   D   L   L   G
1380 CGG CGC ACG CCG CGG CGG GAG GCC ACG CTG GAG CTG CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC

393 C   L   E   D   I   E   E   A   L   C   G   P   A   A   L   P   P   A   P   S   L   L   R
1452 TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCG CTC CCG CCC GCG CCC AGT CTT CTC AGA TGA
1521 GGCTGCGCCCC TGCGGGCAGC TCTAAGGACC GTCCTCGCAG ATCGCCTTCC AACCCCACTT TTTTCTGGAA AGGAGGGGTC
1601 CTTGCAGGGGC AAGCAGGAGC TAGCAGCCGC CTACTTGGTG CTAACCCCTC GATGTACATA GCTTTTCTCA GCTGCCTGCG
1681 CGCCGCCGAC AGTCAGCGCT GTGCGGGCGG AGAGAGGTGC GCCGTGGGCT CAAGAGCCTG AGTGGGTGGT TTGCGAGGAT
1761 GAGGGACGCT ATGCCTCATG CCCGTTTTGG GTGTCCTCAC CAGCAAGGCT GCTCGGGGCG CCCTGGTTCG TCCCTGAGCC
1841 TTTTTCACAG TGCATAAGCA GTTTTTTTTG TTTTTGTTTT GTTTTTAAA TCAATCATGT TACACTAATA
1921 GAAACTTGGC ACTCCTGTGC CCTCTGCCTG GACAAGCAC ATAGCAAGCT GAACTGTCCT AAGGCAGGGG CGAGCACGGA
2001 ACAATGGGGC CTTCAGCTGG AGCTGTGGAC TTTTGTACAT ACACTAAAAT TCTGAAGTTA AG
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Fig.2.

Northern blot (lanes 1-3) of 10 µg of oligo-dT selected RNA from human 293 cells (fibroblast cell line), placenta and spleen hybridised with the TNF receptor cDNA (SmaI-EcoRI fragment). The Southern blot (lanes 4-6) was hybridised with the same probe. Human genomic DNA (5 µg per lane) was digested with PstI(lane 4), Hind 111 (lane 5) and EcoRI (lane 6)
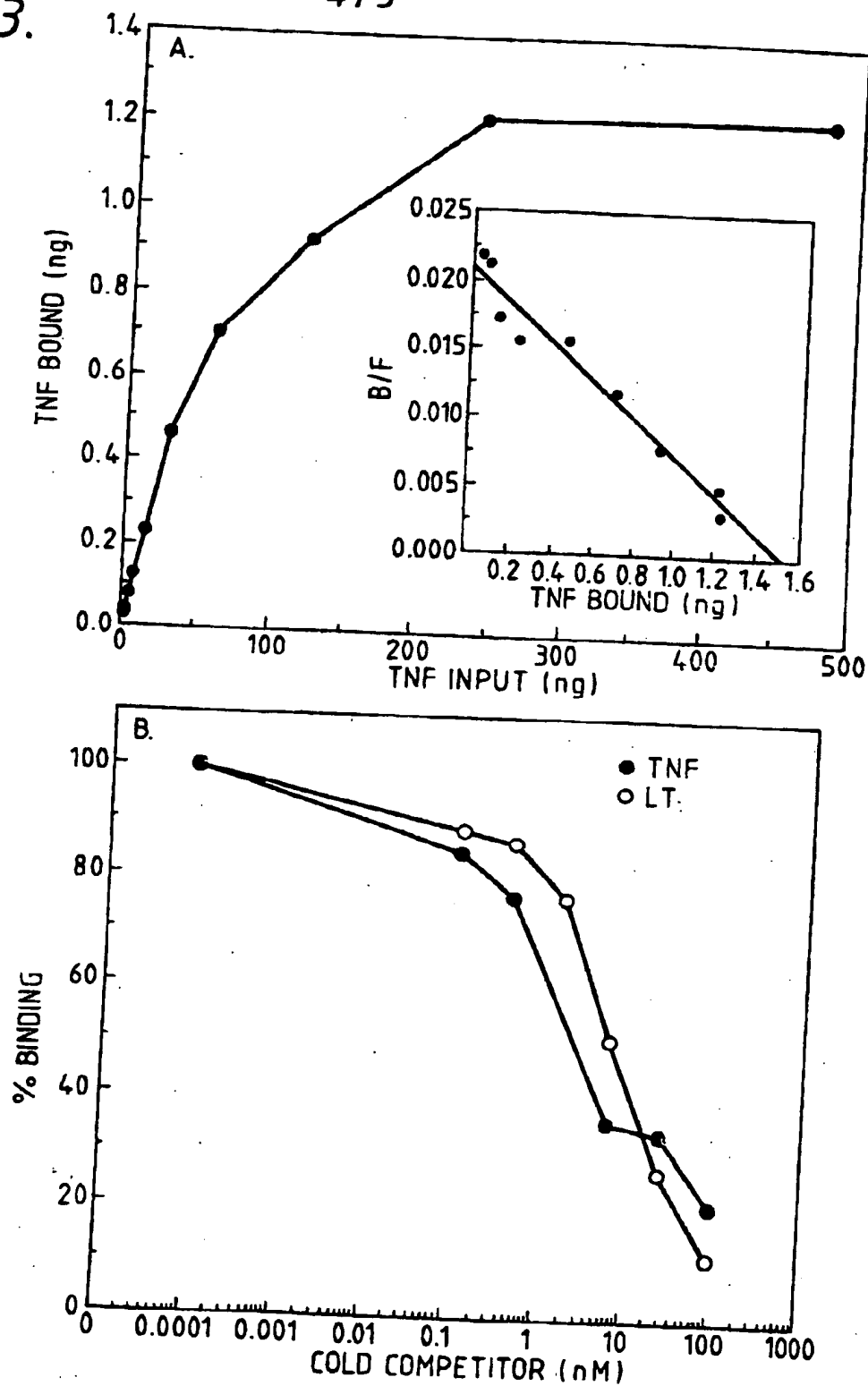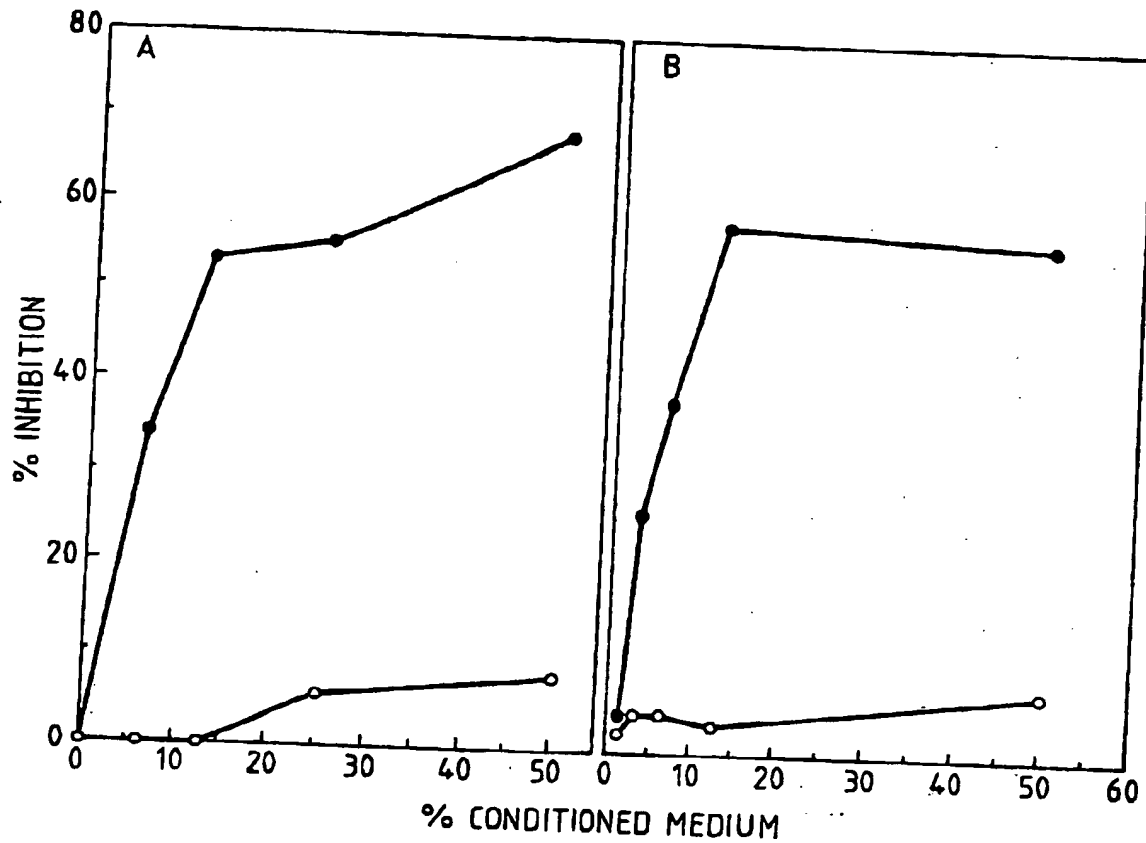
Fig.3.

Fig.4.

Effects of soluble TNF-R on TNF binding and biological activity.
Panel A shows the effects of supernatants from Cos-7 cells transfected
with a cDNA encoding a soluble form of the TNF receptor (pTNFRecd, closed
circles) or mock transfected (open circles) on ^{125}I-TNF binding to U937
cells. Panel B shows the effects of these supernatants on TNF mediated
killing of WEHI 164 (clone 13) line. Assays were performed as described
in Materials and Methods

POLYPEPTIDE AND ITS USE

The present invention relates to recombinant proteins and their use.

Tumour necrosis factor-α (TNFα) is a potent cytokine
5 which elicits a broad spectrum of biological responses. TNFα causes the cytolysis or cytostasis of many tumour cell lines in vitro, induces the haemorrhagic necrosis of transplanted tumours in mice, enhances the phagocytosis and cytotoxicity of polymorphonuclear neutrophils, and
10 modulates the expression of many proteins, including lipoprotein lipase, class I antigens of the major histo-compatibility complex, and cytokines such as interleukin 1 and interleukin 6. TNFα appears to be necessary for a normal immune response, but large quantities produce
15 dramatic pathogenic effects. TNFα has been termed "cachectin" since it is the predominant factor responsible for the wasting syndrome (cachexia) associated with neoplastic disease and parasitemia. TNF is also a major contributor to toxicity in gram-negative sepsis, since
20 antibodies against TNF can protect infected animals.

The many activities of TNFα are mediated by binding to a cell surface receptor. Radioligand binding studies have confirmed the presence of TNF receptors on a wide variety of cell types. Although these receptors are expressed in
25 limited numbers (1,000 - 10,000 receptors/cell), they bind TNFα with high affinity (Ka = $10^9 M^{-1}$ at 4°C). The TNF receptor has been characterized as a 65,000-80,000 dalton glycoprotein which binds both TNFα and the structurally related lymphotoxin (TNFβ). Lymphotoxin has similar, if
30 not identical, biological activities to TNFα, presumably because both are recognized by the same receptor. Recently, several laboratories have detected heterogeneity in TNF receptor preparations, and have proposed that at least two distinct cell surface molecules bind TNFα. In
35 addition, both of these receptors appear to be released

from cells in soluble form, as TNF binding proteins of 30,000 daltons have been isolated from both urine and serum (1-3). This soluble extracellular domain retains the capacity to bind ligand with high affinity.

5   We have now expressed a polypeptide which corresponds to the extracellular domain of a human TNFα receptor. Further, this polypeptide is secreted as a soluble protein and is capable of binding human TNFα. The polypeptide can therefore be used in the treatment of disorders where TNFα

10 has a significant causative role.

Accordingly, the present invention provides a polypeptide having the amino acid sequence (I):

```
     L  V  P  H  L  G  D  R  E  K  R  D  S  V  C  P  Q  G  K  Y
     I  H  P  Q  N  N  S  I  C  C  T  K  C  H  K  G  T  Y  L  Y
15   N  D  C  P  G  P  G  Q  D  T  D  C  R  E  C  E  S  G  S  F
     T  A  S  E  N  H  L  R  H  C  L  S  C  S  K  C  R  K  E  M
     G  Q  V  E  I  S  S  C  T  V  D  R  D  T  V  C  G  C  R  K
     N  Q  Y  R  H  Y  W  S  E  N  L  F  Q  C  F  N  C  S  L  C
     L  N  G  T  V  H  L  S  C  Q  E  K  Q  N  T  V  C  T  C  H
20   A  G  F  F  L  R  E  N  E  C  V  S  C  S  N  C  K  K  S  L
     E  C  T  K  L  C  L  P  Q  I
```

or a derivative thereof to which human TNFα is capable of binding and whose amino acid sequence has a degree of homology of 90% or more with the sequence (I).

25 The invention also provides a DNA sequence which encodes this polypeptide. The DNA sequence may be:

```
     CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT
     CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT
     ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC
30   CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC
     ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA
     TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG
     GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT
     TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC
35   CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC
```

GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT
GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG
TGC CTA CCC CAG ATT

The invention further provides a vector which
5 incorporates a DNA sequence of the invention and which is
capable, when provided in a transformed host, of expressing
the polypeptide of the invention encoded by the DNA
sequence. A host transformed with such a vector forms part
of the present invention too.

10 In the accompanying drawings:

Figure 1 shows the nucleotide sequence of the human TNFα
cDNA and encoded amino acid sequence. The predicted signal
sequence residues are numbered -40 to -1. The
transmembrane domain is boxed and potential N-linked
15 glycosylation sites are overlined. The sequence homologous
with the designed oligonucleotide probe is found at
nucleotide positions 477-533.

Figure 2 is a Northern blot (lanes 1-3) of 10μg of
oligo-dT selected RNA from human 293 cells (fibroblast cell
20 line) (lane 1), placenta (lane 2) and spleen (lane 3)
hybridised with the TNF receptor cDNA (SmaI-EcoRI
fragment). The Southern blot (lanes 4-6) was hybridized
with the same probe. Human genomic DNA (5 μg per lane) was
digested with PstI (lane 4). Hind III (lane 5) and EcoRI
25 (lane 6).

Figure 3 shows the binding characteristics of
recombinant human TNF receptor expressed in COS-7 cells.
The direct binding of recombinant ^{125}I-TNFα to COS-7 cells
transfected with prTNFR is presented in panel A. The inset
30 contains Scatchard analysis derived from this data. As
shown in panel B, monolayers of Cos-7 cells transfected
with TNF-R cDNA were incubated with 1nM ^{125}I-TNF in the
presence of various concentrations of unlabelled TNF or LT.

Figure 4 shows the effects of soluble TNF-R on TNF
35 binding and biological activity. Pan 1 A shows the effects

of supernatants from Cos-7 cells transfected with a cDNA encoding a soluble form of the TNF receptor (pTNFRecd, closed circles) or mock transfected (open circles) on ^{125}I-TNF binding to U937 cells. Panel B shows the effects

5 of these supernatants on TNF mediated killing of WEHI 164 (clone 13) line. Assays were performed as described in Materials and Methods.

In order to obtain a derivative of the polypeptide of the invention, the amino acid sequence shown above may be

10 modified by one or more amino acid substitutions, insertions and/or deletions and/or by an extension at either or each end. A polypeptide composed of such a modified sequence must of course still be capable of binding human TNFα. Typically a modified polypeptide has a

15 binding affinity for human TNFα of 10^7M^{-1} or greater, for example 10^8M^{-1} or greater. The affinity may be from 10^7 to $10^{10}M^{-1}$, for example from 10^8 to 10^9M^{-1}. When the unmodified amino acid sequence shown above is modified there is a degree of homology of 90% or more or 95% or more

20 between the modified and unmodified sequence.

For example, one or more amino acid residues of the sequence above may be substituted or deleted or one or more additional amino acid residues may be inserted; provided the physicochemical character of the original sequence is

25 preserved, i.e. in terms of charge density, hydrophobicity/ hydrophilicity, size and configuration. Candidate substitutions are, based on the one-letter code (Eur. J. Biochem. 138, 9-37, 1984):

A for G and vice versa,

30 V by A, L or G;

K by R;

S by T and vice versa;

E for D and vice versa; and

Q by N and vice versa.

Up to 15 residues may be deleted from the N-terminal of the polypeptide, for example up to 11 residues or up to 5 residues. As far as extensions are concerned, a short sequence of up to 50 amino acid residues may be provided at
5 either or each terminal. The sequence may have up to 30, for example up to 20 or up to 10, amino acid residues. Up to 12 residues, for example up to 9 residues or up to 5 residues, may be added to the C-terminal of the polypeptide in the order E N V K G T E D S G T T.
10 Alternatively, a much longer extension may be present. Longer amino acid sequences may be fused to either or each end. A chimaeric protein may therefore be provided in which the or each extension is a heterologous amino acid sequence, i.e. a sequence not naturally linked to the amino
15 acid sequence above. Such a chimaeric protein may therefore combine the ability to bind specifically to human TNFα with another functionality.

The polypeptides are prepared by recombinant DNA technology. The preparation of the polypeptides therefore
20 depends upon the provision of a DNA sequence encoding the polypeptide. DNA comprising the nucleotide sequence shown above may be obtained by probing a human placenta cDNA library, for example a λgt11 library. Such a library is available from Clontech. A suitable probe is:
25 AAG GAG ATG GGC CAG GTT GAG ATC TCT TCT ACT GTT GAC AAT GAC ACT GTG TGT GGC

A modified form of the nucleotide sequence shown above, a polypeptide having the amino acid sequence shown above or a derivative polypeptide, may be obtained by use of any
30 appropriate technique, including restriction with an endonuclease, insertion of linkers, use of an exonuclease and/or a polymerase and site-directed mutagenesis. Whether a modified DNA sequence encodes a polypeptide of the invention can be readily ascertained. The polypeptide
35 encoded by the sequence can be expressed in a suitable host

and tested for its ability to bind specifically human TNFα.

For expression of a polypeptide of the invention, an expression vector is constructed. An expression vector is prepared which comprises a DNA sequence encoding a
5   polypeptide of the invention and which is capable of expressing the polypeptide when provided in a suitable host. Appropriate transcriptional and translational control elements are provided, including a promoter for the DNA sequence, a transcriptional termination site, and
10 translational start and stop codons. The DNA sequence is provided in the correct frame such as to enable expression of the polypeptide to occur in a host compatible with the vector.

The expression vector is then provided in an appropriate
15 host. Cells harbouring the vector are grown so as to enable expression to occur. The vector may be a plasmid or a viral vector. Any appropriate host-vector system may be employed.

The transformed host may be a prokaryotic or eukaryotic
20 host. A bacterial or yeast host may be employed, for example _E. coli_ or _S. cerevisiae_. Insect cells can alternatively be used, in which case a baculovirus expression system may be appropriate. As a further alternative, cells of a mammalian cell line, such as
25 Chinese Hamster Ovary (CHO) Cells may be transformed. A polypeptide glycosylated at one, two or three of the sites shown in Figure 1 can be obtained by suitable choice of the host cell culture.

The polypeptide of the invention can be isolated and
30 purified. The polypeptide is soluble. It can be employed in the regulation of TNF-mediated responses by binding and sequestering the cytokine. The polypeptide can therefore be used therapeutically to treat disorders such as cachexia, sepsis and autoimmune diseases such as rheumatoid
35 arthritis.

For this purpose, a polypeptide of the present invention may be formulated in a pharmaceutical composition. The pharmaceutical composition also comprises a pharmaceutically acceptable carrier or diluent.

5 The polypeptide of the invention may be administered to a patient by any convenient route. The choice of whether an oral route or a parenteral route, such as subcutaneous, intravenous or intramuscular administration, is adopted; of the dose; and of the frequency of administration depends 10 upon a variety of factors. These factors include the purpose of the administration, the age and weight of the patient being treated and the condition of the patient. Typically, however, the polypeptide is administered in an amount of from 1 to 1000 µg per dose, more preferably from 15 10 to 100 µg per dose, for each route of administration.

The invention further provides a protein which has the amino acid sequence of the extracellular domain of a human TNFα receptor, or a derivative thereof to which human TNFα is capable of binding, for use in a method of treatment of 20 the human or animal body by therapy; especially for use in the treatment of an autoimmune disease in which TNFα plays a significant causative role, such as rheumatoid arthritis.

The extracellular domain of a TNFα receptor, or a derivative thereof capable of binding human TNFα, may 25 therefore be used to treat rheumatoid arthritis. The extracellular domain, or a derivative thereof, of either of the two structurally distinct human TNF receptors may be used. A suitable polypeptide has the amino acid sequence (II):

```
30  D  S  V  C  P  Q  G  K  Y  I  H  P  Q  N  N  S  I  C  C  T
    K  C  H  K  G  T  Y  L  Y  N  D  C  P  G  P  G  Q  D  T  D
    C  R  E  C  E  S  G  S  F  T  A  S  E  N  H  L  R  H  C  L
    S  C  S  K  C  R  K  E  M  G  Q  V  E  I  S  S  C  T  V  D
    R  D  T  V  C  G  C  R  K  N  Q  Y  R  H  Y  W  S  E  N  L
35  F  Q  C  F  N  C  S  L  C  L  N  G  T  V  H  L  S  C  Q  E
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```
K  Q  N  T  V  C  T  C  H  A  G  F  F  L  R  E  N  E  C  V
S  C  S  N  C  K  K  S  L  E  C  T  K  L  C  L  P  Q  I  E
N  V  K  G  T  E  D  S  G  T  T
```

or a derivative thereof to which human TNFα is capable of
binding.

The amino acid sequence of an extracellular domain such
as sequence (II) shown above may be modified by one or more
amino acid substitutions, insertions and/or deletions
and/or by an extension at either or each end. A
polypeptide composed of such a modified sequence must of
course still be capable of binding human TNFα. Typically a
modified polypeptide has a binding affinity for human TNFα
of $10^7 M^{-1}$ or greater, for example $10^8 M^{-1}$ or greater. The
affinity may be from 10^7 to $10^{10} M^{-1}$, for example from 10^8
to $10^9 M^{-1}$. When the unmodified amino acid sequence shown
above is modified there is a degree of homology of 90% or
more or 95% or more between the modified and unmodified
sequence.

For example, one or more amino acid residues of the
sequence above may be substituted or deleted or one or more
additional amino acid residues may be inserted; provided
the physicochemical character of the original sequence is
preserved, i.e. in terms of charge density, hydrophobicity/
hydrophilicity, size and configuration. Candidate
substitutions are, based on the one-letter code (Eur. J.
Biochem. 138, 9-37, 1984):

A for G and vice versa,
V by A, L or G;
K by R;
S by T and vice versa;
E for D and vice versa; and
Q by N and vice versa.

Up to 15 residues may be added to the N-terminal of the
polypeptide, for example up to 11 residues or up to 5
residues. As far as extensions are concerned, a short
sequence of up to 50 amino acid residues may be provided at

either or each terminal. The sequence may have up to 30, for example up to 20 or up to 10, amino acid residues. Up to 12 residues, for example up to 9 residues or up to 5 residues, may be deleted from the C-terminal of the
5 polypeptide.

For this purpose, a polypeptide may be formulated in a pharmaceutical composition. The pharmaceutical composition also comprises a pharmaceutically acceptable carrier or diluent. The polypeptide is typically a recombinant
10 polypeptide in pure form.

The polypeptide may be administered to a patient by any convenient route. The choice of whether an oral route or a parenteral route, such as subcutaneous, intravenous or intramuscular administration, is adopted; of the dose; and
15 of the frequency of administration depends upon a variety of factors. These factors include the purpose of the administration, the age and weight of the patient being treated and the condition of the patient. Typically, however, the polypeptide is administered in an amount of
20 from 1 to 1000 μg per dose, more preferably from 10 to 100 μg per dose, for each route of administration.
The following Example illustrates the invention.

EXAMPLE

1. Materials and Methods
25   Reagents

Recombinant human TNFα and TNFβ were supplied as highly purified proteins derived from E. coli. The specific activities of these preparations were approximately 10^7 units/mg, as measured in the murine L929 cell cytotoxicity
30 assay. The synthetic oligonucleotides were prepared by Oswel DNA Service (University of Edinburgh).

Isolation of TNF receptor cDNA clones
The sequence of a peptide fragment (E M G Q V E I S S T

V D R D T V C G) of the TNF binding protein was used to design a synthetic oligonucleotide probe (5' AAG GAG ATG GGC CAG GTT GAG ATC TCT TCT ACT GTT GAC AAT GAC ACT GTG TGT GGC-3'). The 57-mer DNA probe was labelled with ^{32}P and T4

5 polynucleotide kinase (New England Biolab, Beverly, MA) and used to screen a placenta cDNA library in λgt10 (4,5). Approximately 800,000 phage were transferred to nitrocellulose filters and screened at reduced stringency (6). Filters were incubated for 2 hours at 42°C in 0.05M

10 sodium phosphate, pH 6.5, 20% formamide, 0.75 M sodium chloride, 0.075 M sodium citrate, 1% polyvinyl pyrrolidone (Sigma, St Louis, MO), 1% Ficoll, 1% bovine serum albumin (Sigma), and 50 ng/ml sonicated salmon sperm DNA (Sigma). The radiolabelled probe was then added to the filters (10^8

15 cpm/ml final concentration) which were hybridized for 16 hours. Filters were washed extensively in 0.06M sodium chloride, 0.006M sodium citrate, 1% SDS at 37°C and positive clones were identified by autoradiography. Ten hybridizing clones were plaque purified (4) and cDNA insert

20 size was determined by polyacrylamide gel electrophoresis of EcoRI digested phage DNA. The inserts of two cDNA clones were sequenced using the dideoxy chain termination technique (7).

Southern and Northern blot analysis

25 DNA was isolated from human lymphocytes by the method of Blin and Stafford (8) and used for Southern blot analysis (9). DNA was digested with restriction endonucleases (New England Biolabs), fractionated on a 1% agarose gel, and transferred to nitrocellulose. Hybridization and washing

30 were conducted under stringent conditions (5) using a ^{32}P-labelled preparation of a 600 bp fragment of the TNF receptor cDNA. Northern blot analysis was performed (10) on oligo-dT selected RNA isolated from human placenta, spleen (generously provided by the Cooperative Human Tissue

35 Network, Birmingham, AL) and a fibroblast cell line (293

cells). Following electrophoresis on a formaldehyde 1.2%
agarose gel, the RNA was transferred to nitrocellulose and
hybridized with the TNF receptor DNA probe under stringent
conditions.

5 ## Mammalian cell expression of the human TNF receptor and derivatives

The coding region of the majority of the human TNF
receptor was isolated as an EcoRI fragment and cloned into
a mammalian cell expression vector (11), resulting in
10 plasmid prTNFR. The EcoRI fragment encodes 374 amino acids
of the TNF receptor; the 81 carboxyl terminal residues of
the cytoplasmic domain are therefore missing from this
plasmid construction. A derivative of the TNF receptor was
produced by engineering a termination codon just prior to
15 the transmembrane domain. The polymerase chain reaction
(PCR) technique (12) was used to generate a 300 bp
restriction fragment containing a BgIII site at the 5' end
and a HindIII site preceded by a TAG stop codon at the 3'
end. The PCR primers were 5'GCTGCTCCAAATGCCGAAAG and
20 5'AGTTCAAGCTTTTACAGTGCCCTTAACATTCTAA. The PCR product was
gel purified and cloned into the TNF receptor expression
plasmid (described above) digested with BgIII and HindIII.
DNA sequencing confirmed that the resulting plasmid
(pTNFRecd) contained the designed DNA sequence.
25 The TNF receptor expression plasmids were transfected
into monkey COS-7 cells using Lipofectin (Glbco BRL,
Bethesda, MD) according to the manufacturer's instructions.
Cells were cultured in Dulbecco's modified Eagle's medium
containing 10% fetal calf serum.

30 ## Analysis of recombinant TNF receptor derivatives

TNFα was radioiodinated with the Iodogen method (Pierce)
according to the manufacturer's directions. The specific
activity of the ^{125}I-TNFα was 10-30 μCU μg. COS cells

transfected with the TNF receptor cDNA (prTNFR, 1300 bp EcoRI fragment) were incubated for 24 hours and then seeded into six well tissue culture plates (Nunc) at 4.5 x 10^8 cells per well. The cells were incubated for a further 48
5 hours and then receptor expression was quantitated by radioligand binding for 2 hours at 4°C. Non-specific binding of ^{125}I-TNFα was determined in the presence of a 1,000 fold molar excess of unlabelled TNFα. Binding data was analysed by the method of Scatchard (13).
10 The TNF receptor derivative was analysed for inhibition of ^{125}I-TNFα binding to the natural receptor on human U937 cells. Culture supernatant was harvested 72 hours after COS cells were transfected with pTNFRecd. U937 cells (2 x 10^8 cells in 200 µl) were incubated with 1nM ^{125}I-TNFα and
15 dilutions of COS cell media for 2 hours at 4°C. Cells were then centrifuged through 20% sucrose to remove unbound TNFα. Non specific binding was determined in the presence of 1µM unlabelled TNFα.

The TNF receptor derivative was also analyzed for
20 inhibition of TNFα cytotoxic effects in vitro. The cytotoxicity assay was performed as described on the TNF sensitive cell line WEHI 164 clone 13 (14). Serial dilutions of supernatants from COS cells transfected with pTNFRecd or mock transfected controls were incubated with a
25 constant amount of TNFα (1 ng/ml) for 1 hour at 27°C before addition to the assay.

2. RESULTS

Isolation and characterization of the TNF receptor cDNA
A partial amino acid sequence of the TNF binding protein
30 was used to design a synthetic oligonucleotide probe. The radiolabelled probe was used to screen a human placenta cDNA library in λgt10 and ten hybridizing phage were isolated. The nucleotide and deduced amino acid sequences of the longest cDNA clone are depicted in Figure 1. The

third potential ATG initiation codon occurs at position 156
of the nucleotide sequence; the first two ATG codons are
closely followed by termination codons, and the third ATG
is preceded by the best translation initiation consensus
5 nucleotides (15). The cDNA encodes an open reading frame
of 1365 bases which codes for a polypeptide of 455
residues. Both of the peptide sequences determined by
amino acid sequencing were identified in the encoded cDNA
(17 of 19 and 18 of 19 matching residues). The amino
10 terminal end identified for the TNF binding protein
corresponds to the cDNA encoded sequence beginning at
residue 41. The first 35 amino acids are generally quite
hydrophobic and probably represent a signal sequence.
Residues 35-40 are highly charged (DREKR) and such a
15 sequence is not typically found in secretory signal
sequences (16); perhaps the natural receptor is processed
by proteolysis after residue 40 which contains a dibasic
cleavage site (KR). Hydropathy analysis of the protein
sequence predicts a single transmembrane domain of 23 amino
20 acids. This hydrophobic sequence divides the protein into
an extracellular domain of 171 residues and a cytoplasmic
domain of 221 residues. The amino acid composition
determined for the TNF binding protein (2) corresponds well
with the predicted composition of the extracellular domain
25 encoded by the cDNA (results not shown). The discrepancy
between the predicted receptor size (40,000 daltons) and
the size determined by SDS-polyacrylamide gel
electrophoresis (65,000 daltons, 17-19) is probably due to
glycosylation; there are four potential N-linked
30 glycosylation sites in the sequence, three of which are in
the extracellular domain. The sequence contains a large
number (16) of cysteine residues, 24 of which are in the
extracellular domain. The arrangement of these cysteine is
similar to that of several other cell surface proteins,
35 suggesting that the TNF receptor is structurally related to

a family of receptors.

A Northern blot analysis is presented in Figure 2. The ^{32}P-labelled cDNA hybridized to a single predominant band of oligo-dT selected RNA from human placenta or spleen. A
5 minor larger transcript was also observed in RNA from a fibroblast cell line. The size of the hybridizing species is 2400 bases, in good agreement with the size of isolated cDNA. Also shown in Figure 2 is a Southern blot of human genomic DNA hybridized with a 600 bp probe from the cDNA.
10 In each of the three different restriction digests, only a single hybridized signal was observed. Thus the TNF receptor that we have isolated appears to be encoded by a single gene.

15 <u>Expression of recombinant TNF receptor sequences in mammalian cells</u>

To confirm that the cDNA shown in Figure 1 indeed encodes the TNF receptor, the cDNA was engineered for expression in mammalian cells. The cDNA contains an EcoRI
20 site at position 1270 of Figure 1. The receptor coding sequence was isolated as a 1300 bp EcoRI-fragment (containing all but the last 81 codons of the cytoplasmic domain) and inserted into a mammalian cell expression vector containing a cytomegalovirus promoter and SV40
25 transcription termination sequences (11). The resulting plasmid was transfected into COS cells which were analyzed for TNF receptor expression after three days. As shown in Figure 3, the transfected cells specifically bound radioiodinated TNFα in a saturable and dose dependent
30 fashion. The population of COS cells expressed approximately 1×10^8 receptors per cell. The measured binding affinity of recombinant receptors was $2.5 \times 10^9 M^{-1}$ at 4°C which is in close agreement with natural receptor on human cells (18,19). The binding of ^{125}I-TNFα(1 nM) to
35 these cells could be inhibited by the addition of

unlabelled TNFα or lymphotoxin (Figure 3b). COS cells
transfected with just the expression vector did not
significantly bind ^{125}I-TNFα (less than 2% of the binding
seen with the cDNA transfection).

5   The extracellular domain of the TNF receptor is
naturally shed from cells (1-3). To produce a similar
recombinant derivative, a stop codon preceding the
transmembrane domain was engineered into the cDNA by PCR
mutagenesis. The modified DNA was inserted into the
10   expression plasmid and subsequently transfected into COS
cells. After three days, the COS cell media was tested for
inhibition of TNFα binding to human U937 cells. As shown
in Figure 4a, the transfected cell media inhibited up to
70% of the binding of TNFα. The recombinant TNF receptor
15   derivative was next tested for inhibition of TNFα
biological activity. A sensitive bioassay for TNFα is a
measurement of cytolysis of mouse WEHI 164 (clone 13)
cells. The transfected cell media inhibited 60% of TNFα
cytotoxicity on this cell line (Figure 4b). Media from
20   mock transfected COS cells did not inhibit TNFα induced
cytotoxicity or binding. These experiments demonstrate
that the recombinant extracellular domain of the TNF
receptor is capable of binding TNF and inhibiting its
biological activity.

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CLAIMS

1. A polypeptide having the amino acid sequence:

```
   L  V  P  H  L  G  D  R  E  K  R  D  S  V  C  P  Q  G  K  Y
   I  H  P  Q  N  N  S  I  C  C  T  K  C  H  K  G  T  Y  L  Y
 5 N  D  C  P  G  P  G  Q  D  T  D  C  R  E  C  E  S  G  S  F
   T  A  S  E  N  H  L  R  H  C  L  S  C  S  K  C  R  K  E  M
   G  Q  V  E  I  S  S  C  T  V  D  R  D  T  V  C  G  C  R  K
   N  Q  Y  R  H  Y  W  S  E  N  L  F  Q  C  F  N  C  S  L  C
   L  N  G  T  V  H  L  S  C  Q  E  K  Q  N  T  V  C  T  C  H
10 A  G  F  F  L  R  E  N  E  C  V  S  C  S  N  C  K  K  S  L
   E  C  T  K  L  C  L  P  Q  I
```

or a derivative thereof to which human TNFα is capable of binding and whose amino acid sequence has a degree of homology of 90% more with the sequence (I).

15 2. A DNA sequence which encodes a polypeptide as defined in claim 1.

3. A DNA sequence according to claim 2, which is:

```
   CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT
   CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT
20 ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC
   CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC
   ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA
   TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG
   GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT
25 TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC
   CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC
   GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT
   GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG
   TGC CTA CCC CAG ATT
```

30 4. A DNA sequence according to claim 3, which further comprises a 5' sequence which encodes a signal amino acid sequence.

5. A vector which incorporates a DNA sequence as claimed in any one of claims 2 to 4 and which is capable, 35 when provided in a suitable host, of expressing the said

polypeptide.

6. A vector according to claim 5, which is a plasmid.

7. A host transformed with a vector as claimed in claim 5 or 6.

8. A host according to claim 7, which is a mammalian cell line.

9. A process for the preparation of a polypeptide as defined in claim 1, which process comprises culturing a transformed host as claimed in claim 7 or 8 under such conditions that the said polypeptide is expressed.

10. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as an active principle, a polypeptide as claimed in claim 1.

11. A protein which has the amino acid sequence of the extracellular domain of a human TNFα receptor, or a derivative thereof to which human TNFα is capable of binding, for use in a method of treatment of the human or animal body by therapy.

12. A protein according to claim 11, for use in the treatment of rheumatoid arthritis.

13. A protein according to claim 11 or 12, which is a polypeptide as defined in claim 1.